Supporting Online Material

Materials and Methods

Materials. Oligonucleotides were either purchased from Integrated DNA Technologies (San Diego, CA) or synthesized on an Expedite automated DNA/RNA synthesizer (Applied Biosystems, Foster City, CA) using nucleoside phosphoramidites purchased from Glen Research (Sterling, VA). All oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis (PAGE) and desalted using a C18 SEP-Pak cartridge (Waters, Milford, MA). Histidine-tagged T7 RNA polymerase was purified from *E. coli* strain BL21 containing plasmid pBH161 (kindly provided by William McAllister, State University of New York, Brooklyn). *Thermus aquaticus* DNA polymerase was cloned from total genomic DNA and purified as previously described [F. G. Pluthero, *Nucleic Acids Res.* **21,** 4850 (1993)]. M1 RNA, the catalytic subunit of RNAse P, was obtained from *E. coli* genomic DNA (Sigma-Aldrich, St. Louis, MO) by PCR amplification using primers 5´-GGACTAATACGACTCACTATAGAAGCTGACCAGACAGTCG-3´ and 5´-AGGTGAAACTGACCGATAAGC-3 (T7 RNA polymerase promoter sequence underlined), followed by *in vitro* transcription. The PCR products were cloned into *E. coli* and their sequence was verified, as described below. Calf intestine phosphatase, *E. coli* poly(A) polymerase, and T4 polynucleotide kinase were purchased from New England Biolabs (Ipswich, MA), Superscript II RNase H^- reverse transcriptase was from Invitrogen (Carlsbad, CA), and calf thymus terminal transferase was from Roche Applied Science (Indianapolis, IN). Nucleoside and deoxynucleoside 5´-triphosphates were purchased from Sigma-Aldrich and [γ-^{32}P]ATP (7 μCi/pmol) was from Perkin Elmer (Waltham, MA).

Preparation of RNA enzymes and substrates. All RNA enzymes and substrates were prepared by *in vitro* transcription. The transcription mixture contained 0.4 μM DNA template, 0.8 μM synthetic oligodeoxynucleotide having the sequence 5´-GGACTAATACGACTCACTATA-3´ (promoter sequence underlined), 2 mM each of the four NTPs, 25 U/μL T7 RNA polymerase, 15 mM $MgCl_2$, 2 mM spermidine, 5 mM dithiothreitol, and 50 mM Tris-HCl (pH 7.5). The mixture was incubated at 37 °C for 2 h, then quenched by adding an equal volume of gel loading buffer containing 15 mM Na_2EDTA and 18 M urea. The transcription products were purified by PAGE, eluted from the gel, and desalted.

The A substrates could not be obtained reliably by *in vitro* transcription due to heterogeneity at the 3′ end of the transcripts. Instead, extended length RNAs were prepared that contained additional nucleotides, having the sequence 5′-GAGACCGCAACUUG-3′, located downstream from the A substrate sequence. The added nucleotides were removed using *E. coli* M1 RNA to generate a precise 3′ terminus. The cleavage reaction employed 20 μM RNA transcript, 20 μM external guide sequence RNA having the sequence 5′-GGUAAGUUGCGGUCUCACCA-3′, 5 μM M1 RNA, 100 mM $MgCl_2$, 100 mM NH_4Cl, and 50 mM Tris-HCl (pH 7.5). Note that the guide RNA is complementary to the extended portion of the transcript, with a 5′-terminal GG and 3′-terminal ACCA also present in the guide RNA [A. C. Forster, S. Altman, *Science* **249,** 783 (1990)]. The reaction mixture was incubated at 30 °C for 8 h, quenched, and the cleaved products were purified by PAGE, as described above. During the *in vitro* evolution procedure, the A′ substrates were prepared directly by *in vitro* transcription, but in all other instances these substrates were prepared using the M1 RNA cleavage procedure. For the A′ substrates, the added 3′-terminal nucleotides had the sequence 5′-GAGACCGCAUGAAU-3′ and the external guide sequence RNA had the sequence 5′-GGAUUCAUGCGGUCUCACCA-3′.

***In vitro* evolution.** DNA templates used to transcribe the starting pools of B-E′ and B′-E molecules were generated by a 10-cycle PCR employing two overlapping synthetic oligodeoxynucleotides, as listed below (promoter sequence underlined; nucleotides randomized at 12% degeneracy in italics). The resulting PCR products, each consisting of ~10^{14} molecules, were transcribed as described above, except that it was unnecessary to provide a synthetic oligodeoxynucleotide containing the second strand of the promoter.

For B-E′

5′-GGAC<u>TAATACGACTCACTATA</u>GAGACCGCAACTTAG-3′ and

5′-GACAGATCAGTATTCATGCGGTCTC*TAAATTCAACCCATTCAAACTGTT-CTAAGTTACCTTAGAACAATCGAGCACA*ACTTACTAAGTTGCGGTCTC-3′;

For B′-E

5′-GGAC<u>TAATACGACTCACTATA</u>GAGACCGCATGAATAG-3′ and

5′-CTTCTGGATGGTCAAGTTGCGGTCTC*TTTATTCAACCCATTCAAACTGTT-ACTTACGTAACAATCGAGCACA*TGAACACTATTCATGCGGTCTC-3′.

DNA templates used to transcribe the starting pools of A and A´ molecules were prepared directly as synthetic oligodeoxynucleotides (promoter sequence underlined; nucleotides randomized at 12% degeneracy in italics). The second strand of the promoter was supplied as a synthetic oligodeoxynucleotide. The transcribed A molecules were cleaved by M1 RNA, as described above.

For A

5´-CAAGTTGCGGTCTC*TTTATTCAACCCATTCAAACTGTTACTTACGTAACAATCGAGC-ACA*TGAACTCGTGTTAGCCTATAGTGAGTCGTATTAGTCC-3´;

For A´

5´-*TAATTCAACCCATTCAAACTGTTCTAAGTTACCTTAGAACAATCGAGC-ACA*ACTTCAGCATAGGATTCTATAGTGAGTCGTATTAGTCC-3´.

During each round of *in vitro* evolution, RNA-catalyzed RNA ligation was carried out in a reaction mixture containing 1 μM B-E´ (or B´-E), 5 μM A (or A´), 25 mM $MgCl_2$, and 50 mM EPPS (pH 8.5), which was incubated at 30 °C for various times. The ligated RNAs were gel purified, then reverse transcribed in a reaction mixture containing ~0.4 μM RNA, 1 μM cDNA primer, 0.5 mM each of the four dNTPs, 3 mM $MgCl_2$, 75 mM KCl, 10 mM dithiothreitol, and 50 mM Tris-HCl (pH 8.3), which was incubated at 37 °C for 1 h. The resulting cDNAs were PCR amplified employing the same cDNA primer and a second primer, as listed below (promoter sequence underlined).

For A-B-E´

5´-GACAGATCAGTATTCATGC-3´ and

5´-GGACTAATACGACTCACTATAGGCTAACACGAGTTCA-3´;

For A´-B´-E

5´-CTTCTGGATGGTCAAGTTGC-3´ and

5´-GGACTAATACGACTCACTATAGAATCCTATGCTGAAGT-3´.

The PCR products were used to initiate nested PCR amplifications to generate templates for the transcription of progeny RNAs. For the B-E´ molecules, the products of this second PCR were transcribed directly. For the A molecules, it was necessary to perform three successive PCRs, rather than progressing directly from A-B-E´ to A, due to mispriming caused by sequence similarity near the 3´ ends of A and E´. The second PCR eliminated the 3´-terminal region of E´,

allowing subsequent amplification of A. The products of the second PCR were incubated in the presence of 0.2 N NaOH for 20 min at 92 °C to bring about hydrolysis at the single ribonucleotide position, followed by neutralization with 0.2 N HCl. The shorter cleaved products were purified by PAGE and used as input for the third PCR. The products of the third PCR were transcribed to generate RNA, which was gel purified and cleaved by M1 RNA, as described above. The primers used for the various nested PCRs derived from A-B-E´ are listed below (T7 promoter underlined; ribonucleotide in bold).

For B-E´ (second PCR)
5´-GACAGATCAGTATTCATGC-3´ and
5´-GGACTAATACGACTCACTATAGAGACCGCAACTTAG-3´;

For A (second PCR)
5´-GACAGATCAGTATTCATGC(**rG**)-3´ and
5´-GGACTAATACGACTCACTATAGGCTAACACGAGTTCA-3´;

For A (third PCR)
5´-CTAAGTTGCGGTCTC-3´ and
5´-GGACTAATACGACTCACTATAGGCTAACACGAGTTCA-3´.

For the B´-E molecules, the products of the second PCR were transcribed directly. For the A´ molecules, the products of the second PCR were subjected to alkaline hydrolysis as described above, then the cleaved products were purified by PAGE and used as input for a third PCR. The products of the third PCR also were subjected to alkaline hydrolysis, the cleaved products were purified by PAGE, then used to transcribe the desired A´ molecules. The primers used for the various nested PCRs derived from A´-B´-E are listed below (T7 promoter underlined; ribonucleotide in bold).

For B´-E (second PCR)
5´-CTTCTGGATGGTCAAGTTGC-3´ and
5´-GGACTAATACGACTCACTATAGAGACCGCATGAATAG-3´;

For A´ (second PCR)
5´-CTTCTGGATGGTCAAGTTGC(**rG**)-3´
5´-GGACTAATACGACTCACTATAGAATCCTATGCTGAAGT-3´;

For A´ (third PCR)

5´-CTATTCATGCGGTCT(**rC**)-3´ and

5´-GGACTAATACGACTCACTATAGGAAAGAGAAAGAAGT-3´.

Six successive rounds of *in vitro* evolution were carried out as described above, with progressively shorter times for the RNA-catalyzed reaction:

Round	A-B-E´	A´-B´-E
1	2 h	2 h
2	1 min	5 min
3	15 s	30 s
4	15 s	15 s
5	0.1 s	0.1 s
6	0.01 s	0.01 s

The last two rounds were conducted using a KinTek (Austin, TX) model RQF-3 quench-flow apparatus to achieve very short reaction times. Hypermutagenic PCR [J.-P. Vartanian, M. Henry, S. Wain-Hobson, *Nucleic Acids Res*. **24,** 2627 (1996)] was performed following round 3 to increase diversity among the population of B-E´, B´-E, and A molecules. Standard mutagenic PCR [R. C. Cadwell, G. F., Joyce, *PCR Methods Applic*. **2,** 28 (1992)] was performed following round 3 for the A´ molecules.

Following round 6, the ligated molecules were gel purified, reverse transcribed, PCR amplified, and cloned into *E. coli* using the Invitrogen TOPO TA Cloning Kit. The bacteria were grown on LB agar plates containing 50 μg/ml carbenicillin. Samples were taken from individual colonies and evaluated by PCR to confirm they contained plasmid DNA with an insert of the appropriate length. Validated colonies were picked from the plate and cultured overnight in 2 mL LB medium containing 50 μg/ml carbenicillin. The plasmid DNA was isolated from the cells using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA), then sequenced by Genewiz Inc. (La Jolla, CA).

Conversion of selected enzymes to corresponding substrates. A modified version of the nested PCR amplification procedure described above can be used to produce A and B molecules from corresponding E molecules, and to produce A´ and B´ molecules from corresponding E´ molecules. In this case, B and B´ are produced as separate molecules, rather than joined to E´ and

E, respectively. This requires installing a primer binding site at the 3´ end of B and B´, which also encodes a recognition sequence for the "10-23" RNA-cleaving DNA enzyme [S. W. Santoro, G. F. Joyce, *Proc. Natl. Acad. Sci. USA* **94,** 4262 (1997)]. Cleavage by the DNA enzyme is used to generate transcription products with a precise 3´ terminus [A. M. Pyle, V. T. Chu, E. Jankowsky, M. Boudvillain, *Methods Enzymol.* **317,** 140 (2000)]. A and A´ are produced as above, except that they are derived from PCR-amplified E and E´, rather than A-B-E´ and A´-B´-E, respectively. In addition, the primer binding site at the 5´ end of A and A´ is shifted upstream so as not to encroach on the genotype region of these molecules.

The ligated products E and E´ are purified by PAGE, reverse transcribed, and PCR amplified, as above. A second PCR is carried out to generate templates that are used to transcribe precursor substrates that contain additional nucleotides at their 3´ terminus. The added nucleotides are removed from A and A´ using M1 RNA, as described above. The added nucleotides are removed from B and B´ using a DNA enzyme. The downstream sequences for the various substrates and corresponding external guide sequence RNA or corresponding DNA enzyme are listed below (dot indicates the site for DNA-catalyzed RNA cleavage; substrate-binding domains within the DNA enzyme are underlined).

For A
additional nucleotides 5´-GAGACCGCAAGACCCCCCAG-3´,
guide RNA 5´-GGUCUUGCGGUCUCACCA-3´;

For A´
additional nucleotides 5´-GAGACCGCAUCUGAGACGAUGU-3´,
guide RNA 5´-GGCAGAUGCGGUCUCACCA-3´;

For B
additional nucleotides 5´-AGACCCCCCAG•UACACACACC-3´,
DNA enzyme 5´-GGTGTGTGTAGGCTAGCTACAACGATGGGGGGTCT-3´;

For B´
additional nucleotides 5´-UCUGAGACGAUG•UUGAAAAGAGAG-3´,
DNA enzyme 5´-CTCTCTTTTCAAGGCTAGCTACAACGAATCGTCTCAGT-3´.

DNA-catalyzed cleavage is carried out in a reaction mixture containing 10 μM RNA, 30 μM DNA enzyme, 25 mM $CaCl_2$, and 30 mM EPPS (pH 7.5), which is heated to 70 °C for 2 min, then

incubated at 37 °C for 45 min. Following RNA- or DNA-catalyzed cleavage, the desired products are purified by PAGE.

Serial transfer experiments. Reaction mixtures for exponential amplification of cross-replicating RNAs contained 5 μM each of the A, A´, B, and B´ substrates, 15 or 25 mM $MgCl_2$, and 50 mM EPPS (pH 8.5), which were incubated at 42 °C. The first reaction mixture in a serial transfer experiment contained 0.1 μM each of E and E´, but all subsequent mixtures contained only the E and E´ molecules that were carried over in the transfer. When multiple cross-replicating RNAs were employed, each was present at 0.1 μM concentration in the first reaction mixture, and 5 μM each of the component substrates were present in all of the reaction mixtures. The experiment involving 12 pairs of cross-replicating enzymes was pre-initiated by amplifying each cross-replicator in isolation for 10 h, determining the concentrations of E and E´ that had been produced, and employing an aliquot from these mixtures containing a total of 0.2 μM enzymes to initiate the first reaction of the serial transfer procedure. The enzymes E11 and E11´ amplified so poorly that in their case 0.1 μM of each enzyme was employed directly. The pre-initiation procedure was carried out so that the first reaction of the serial transfer would more closely resemble subsequent reactions with regard to the relative amounts of the two members of a cross-replicating pair (fig. S1B). The enzyme E12´ formed a (5´-UAUG-3´)•(5´-AUAC-3´) mismatch with the A12 substrate, but there was no mismatch between E12 and B12´.

In order to prepare the products of a serial transfer experiment for cloning and sequencing, the E and E´ molecules were purified by PAGE, then 3´-polyadenylated, reverse transcribed, and tailed at the 3´ end of the cDNA using terminal transferase. The polyadenylation reactions contained ~0.4 μM E (or E´), 0.1 U/μL poly(A) polymerase, 0.5 mM ATP, 10 mM $MgCl_2$, 250 mM NaCl, and 50 mM Tris-HCl (pH 8.0), which was incubated for 2 h at 37 °C. The polymerase was extracted with phenol/chloroform, the mixture was desalted using a NAP column (GE Healthcare, Piscataway, NJ), and the extended RNAs were reverse transcribed as described above, using a DNA primer having the sequence 5´-T_{24}V-3´ (V = A, C or G). Full-length cDNAs were purified by PAGE, then extended in a reaction mixture containing ~0.2 μM cDNA, 8 U/μL terminal transferase, 1 mM dGTP, 2.5 mM $CoCl_2$, 200 mM potassium cacodylate, 0.25 mg/ml BSA, and 25 mM Tris-HCl (pH 6.6), which was incubated at 37 °C for 2 h. The proteins were extracted with phenol/chloroform, the mixture was desalted using a NAP column, and the extended

cDNAs were PCR amplified using primers having the sequence 5′-GACAGATCAGT$_{24}$V-3′ and 5′-GGCTAACACGAC$_{14}$G-3′. The PCR products were cloned and sequenced, as described above.

Kinetic analysis. RNA-catalyzed RNA ligation was carried out in a reaction mixture containing 5 μM E (or E′), 0.1 μM [5′-^{32}P]-labeled A′ (or A), 6 μM B′ (or B), 15 or 25 mM $MgCl_2$, and 50 mM EPPS (pH 8.5), which was incubated at 30 °C. The reaction was initiated by mixing equal volumes of two solutions, one containing the enzymes and substrates, and the other containing the $MgCl_2$ and EPPS buffer. Aliquots were taken at various times and quenched by adding an equal volume of gel-loading buffer containing 25 mM Na_2EDTA and 18 M urea. The products were separated by PAGE and quantitated using a PharosFX molecular imager (Bio-Rad, Hercules, CA). The data were fit to the equation:

$F_t = a(1 - e^{-kt}) + b$, where F_t is the fraction reacted at time t, a is the maximum extent of the reaction (typically 0.88–0.92), k is the observed rate of product formation, and b is the calculated extent at t = 0 (typically 0.01–0.03).

Reactions catalyzed by E7′, E11, and E11′ were so slow that the data instead were fit to the linear equation: $F_t = at + b$.

Cross-catalytic exponential amplification was carried out in a reaction mixture containing 0.1 μM each E and E′, 5 μM each [5′-^{32}P]-labeled A and A′, 5 μM each B and B′, 15 or 25 mM $MgCl_2$, and 50 mM EPPS (pH 8.5), which was incubated at 42 °C. The reaction was initiated as described above. Aliquots were taken at various times, quenched, and the amounts of newly-synthesized E and E′ were quantitated as described above. The data were fit to the logistic growth equation, as described in the main text.

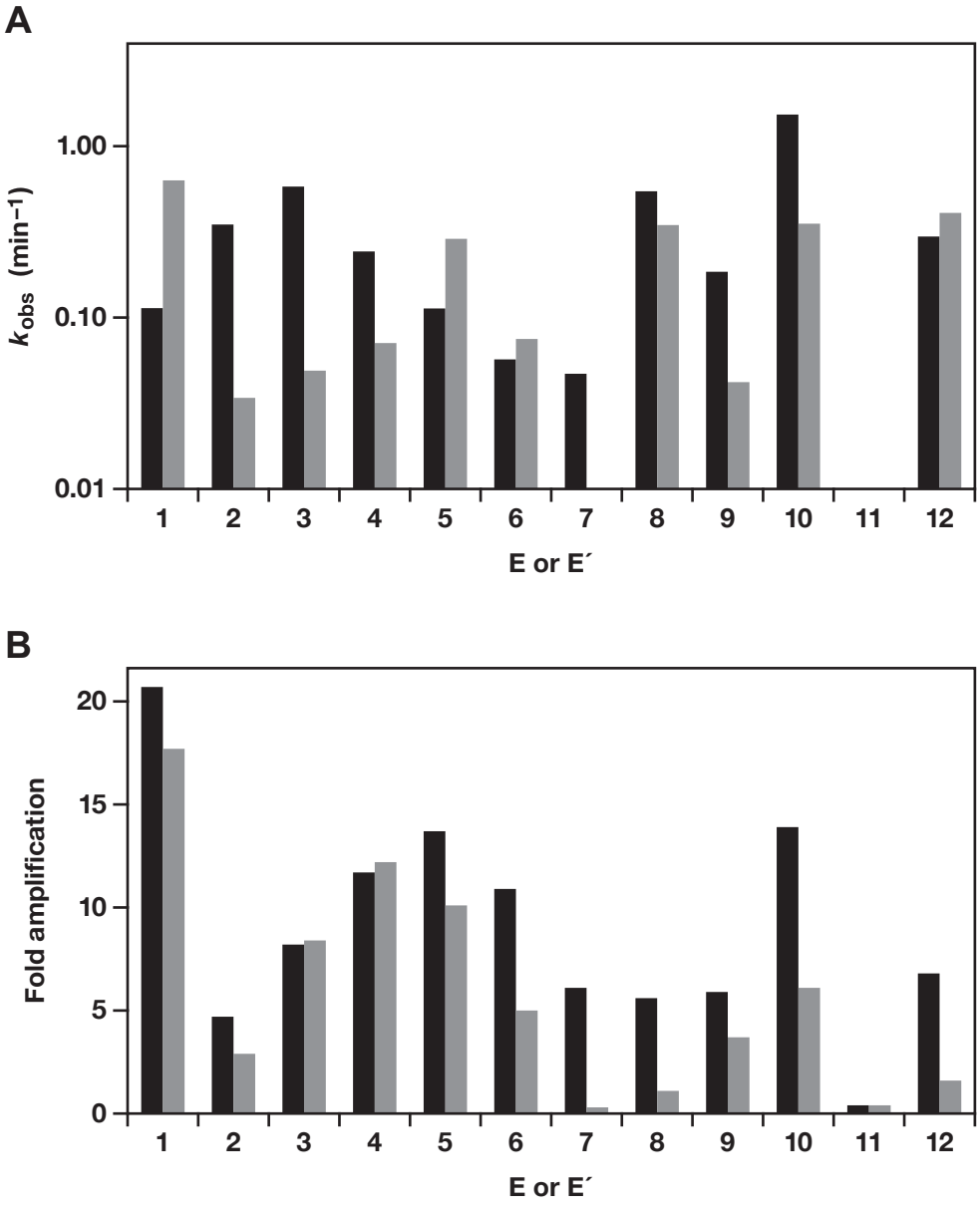
Figure Legends

Fig. S1. Catalytic activity and exponential amplification of 12 pairs of cross-replicating RNA enzymes (Fig. 3). (**A**) For each pair, the observed rate of E (black) and E′ (gray) was measured in a reaction mixture containing 5 μM E (or E′), 0.1 μM [5′-^{32}P]-labeled A′ (or A), 6 μM B′ (or B), 15 mM $MgCl_2$, and 50 mM EPPS (pH 8.5), which was incubated at 30 °C. Values for k_{obs} were determined as described above. (**B**) For exponential amplification, the yield of newly-synthesized

E and E´ relative to the starting amount of each enzyme was determined following incubation at 42 °C for 5 h in a reaction mixture containing 0.1 μM each E and E´, 5 μM each [5´-^{32}P]-labeled A and A´, 5 μM each B and B´, 15 mM $MgCl_2$, and 50 mM EPPS (pH 8.5).

Fig. S2. Serial transfer experiment initiated by cross-replicating RNA enzymes E1–E4 and their partners E1´–E4´ (Fig. 3). (**A**) Amplification was sustained for 16 successive rounds of ~20-fold amplification and 20-fold dilution. The concentrations of all E (black) and E´ (gray) molecules were measured at the end of each incubation. (**B**) Observed genotypes among 25 E´ clones that were sequenced following the last incubation. (**C**) Estimated $\Delta\Delta G$ values for binding of each possible combination of A•B´, A•B, A´•B´, A´•B pairings relative to the corresponding matched interaction (dashes). It is difficult to calculate ΔG values in the context of the enzyme-substrate complex, but $\Delta\Delta G$ values only consider relative predicted binding energy for the paired region, based on values obtained from the mfold web server at Rensselaer Polytechnic Institute [D. H. Mathews, J. Sabina, M. Zuker, D. H. Turner, *J. Mol. Biol.* **288,** 911 (1999); M. Zuker, *Nucleic Acids Res.* **31,** 3406 (2003)]. $\Delta\Delta G$ values that are <3.5 kcal/mol are highlighted in red. (**D**) Preferred pathways for mutation among B (and B´) substrates and among A´ substrates, corresponding to the most favorable $\Delta\Delta G$ values for mismatched pairings shown in fig. S2C.

Figure S1


A
1.00
0.10
0.01
k_{obs} (min^{-1})
1
2
3
4
5
6
7
8
9
10
11
12
E or E´
B
20
15
10
5
0
Fold amplification
1
2
3
4
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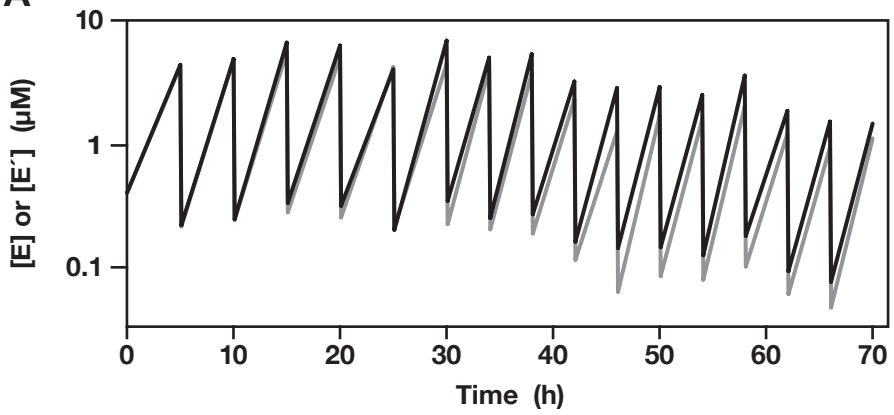
7
8
9
10
11
12
E or E´

Figure S2

A



B

25 clones

B´ \ A´	1	2	3	4
1	2	1	1	2
2	2	3		
3	2	2	2	
4	3	2	2	1

C

ΔΔG values (kcal/mol)

	B1´	B2´	B3´	B4´	B1	B2	B3	B4
A1	—	6.5	6.2	5.0	5.8	5.7	6.7	5.3
A2	6.0	—	4.8	6.8	6.2	6.8	6.5	4.7
A3	5.4	4.6	—	5.5	6.5	6.5	6.5	4.5
A4	5.4	6.8	6.1	—	6.1	4.9	5.2	5.6
A1´	5.5	3.1	4.9	3.1	—	4.5	4.8	4.9
A2´	4.1	3.9	5.7	4.0	4.9	—	4.1	5.7
A3´	5.4	5.4	4.3	3.2	4.8	3.4	—	3.0
A4´	3.8	3.1	4.5	3.7	3.2	5.2	5.0	—

D